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ORIGINAL PAPER

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A *Pichia pastoris* *VPS15* homologue is required in selective peroxisome autophagy

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Abstract Methylophilic yeasts contain large peroxisomes during growth on methanol. Upon exposure to excess glucose or ethanol these organelles are selectively degraded by autophagy. Here we describe the cloning of a *Pichia pastoris* gene (Pp*VPS15*) involved in peroxisome degradation, which is homologous to *Saccharomyces cerevisiae* *VPS15*. In methanol-grown cells of a *P. pastoris* *VPS15* deletion strain, the levels of peroxisomal marker enzymes remained high after addition of excess glucose or ethanol. Electron microscopic studies revealed that the organelles were not taken up by vacuoles, suggesting that Pp*VPS15* is required at an early stage in peroxisome degradation.

Key words *Pichia pastoris* · Peroxisome degradation · Autophagy · *VPS15*

Introduction

Microbodies (peroxisomes, glyoxysomes) are essential compartments of eukaryotic cells (Subramani 1993; Lazarow and Moser 1995). Although simple in structure, their metabolic repertoire is remarkably versatile. A characteristic of peroxisomes is their inducibility. Of

all eukaryotes, the highest peroxisome induction levels are observed in methylophilic yeasts. In these organisms the peroxisomal-volume fraction may exceed 80% of the total cell volume during growth of cells in methanol-limited continuous cultures (Van der Klei et al. 1991 a). Under these conditions peroxisomes are essential for growth because they contain key enzymes of both methanol dissimilation [alcohol oxidase (AO), catalase] and assimilation (dihydroxyacetone synthase).

Methanol-induced peroxisomes are actively degraded when the cells are shifted to conditions in which the organelles are redundant for growth (e.g. when exposed to excess glucose or ethanol). In *Hansenula polymorpha* peroxisome degradation is a rapid and selective process that is basically similar to the macroautophagy described for mammalian cells (Veenhuis et al. 1993).

Remarkably, in the related yeast *Pichia pastoris* two modes of selective peroxisome degradation have been described, which occur dependent on the substrate by which peroxisome autophagy is induced. Exposure of methanol-grown *P. pastoris* cells to ethanol results in degradation by a mechanism similar to that described above for *H. polymorpha* (macroautophagy). Glucose-induced degradation, however, results in the uptake of clusters of peroxisomes in the central vacuole after engulfment by protrusions extending from this organelle. The latter process is analogous to the microautophagy described for mammalian cells (Tuttle and Dunn 1995; Sakai et al. 1998).

Numerous *H. polymorpha* and *P. pastoris* mutants affected in the selective degradation of peroxisomes have been isolated in different laboratories (Titorenko et al. 1995; Tuttle and Dunn 1995; Sakai et al. 1998). We have isolated *H. polymorpha* mutants affected in peroxisome degradation (pdd mutants) and used these to clone the genes involved in this process (*PDD* genes). One mutant, called pdd1, was affected in an early stage of selective peroxisome degradation, namely the envelopment of the organelles within membranes. The protein encoded by Hp*PDD1* (Kiel et al. 1999), showed high sequence similarity to the *Saccharomyces cerevisiae* *VPS34* product,

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ScVps34p (Herman and Emr 1990). This led us to investigate whether a homologue of Vps15p, which is known to be essential for the activation of Vps34p, may also be involved in selective peroxisome degradation in methylotrophic yeasts.

In this paper we describe the cloning of a putative *VPS15* homologue from *P. pastoris* (Pp*VPS15*) and the construction and use of a *P. pastoris* strain deleted for the gene. We show that this gene is indeed essential for an early step in selective peroxisome degradation in *P. pastoris*.

Materials and methods

Strains, media and microbial techniques. *P. pastoris* strains used in this study were GS200 (*his4 arg4*), GS115 (*his4*) (Waterham et al. 1996) and OP5 (*VPS15D::ARG4 his4 arg4*). The cells were grown at 30 °C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or a minimal medium (0.17% w/v yeast nitrogen base without amino acids and without ammonium sulphate (Difco, Detroit, Mich., USA) supplemented with either 0.4% (w/v glucose, YND), 0.5% methanol (v/v, YNM) or 1% ethanol (v/v, YNE) as carbon sources in the presence of 0.25% w/v ammonium sulphate as a nitrogen source. Amino acids were added to a final concentration of 50 mg/ml as required. Cultivation of *Escherichia coli* DH5a was performed essentially as described previously (Sambrook et al. 1989).

Isolation and sequence analysis of Pp*VPS15*. A portion of the Pp*VPS15* gene (3358 bp) was identified on a previously cloned DNA fragment adjacent to the *P. pastoris* *PEX2* gene in plasmid pYT6 (Waterham et al. 1996). The remaining 3' terminal sequences of Pp*VPS15* were isolated by two successive rounds of chromosome walking using the polymerase chain reaction (PCR). In each round of the PCR, a primer complementary to the 3'-most cloned sequences from the gene and another primer complementary to sequences in the *P. pastoris* genomic library vector pYM8 (Liu et al. 1995) were added to amplification reactions along with *P. pastoris* genomic library DNA as a template and either *Taq* (Gibco BRL, Gaithersburg, Md., USA) or *Vent* (NEB, Beverly, Mass., USA) polymerases. Primers used in this study are shown in Table 1. The first round of PCR chromosome walking (PCR#1) using primers 8 and 10 generated a DNA fragment of 774 bp that contained 133 bp from pYT6 and 507 bp of an additional 3' Pp*VPS15* sequence (Fig. 1 A). The second round of PCR amplification (PCR#2) using primers 9 and 10 resulted in a DNA fragment of 880 bp that contained 150 bp in common with the PCR#1 fragment and 596 bp of additional *P. pastoris* DNA sequence including the remaining 158 bp of the Pp*VPS15* ORF. To avoid potential PCR-amplification mistakes, at least two independently amplified fragments were sequenced. For analysis of DNA and

amino-acid sequences MacVector software (IBI, New Haven, Conn., USA) was used. Sequence alignments were performed using the Clustal-W version 1.6 algorithm (Thompson et al. 1994). The BLAST Network Service of the National Center for Biotechnology Information (Bethesda, Md., USA) was used to search for amino-acid sequence similarities. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). DNA sequencing was performed at the Oregon Regional Primate Research Center, Molecular Biology Core Facility (Beaverton, Ore., USA).

Construction of a Pp*VPS15* deletion strain. A vector capable of deleting most of the Pp*VPS15* ORF was constructed in three steps. In the first step, a 2.7-kb *HindIII*/*SalI* fragment from pYM25 (Thompson et al. 1994) containing the *S. cerevisiae* *ARG4* gene was inserted into *HindIII*- and *SalI*-digested pBluescript SKII to create pOS5. In the second step, a 1035-bp fragment containing sequences just 5' of the methionine initiator ATG of Pp*VPS15* was amplified by PCR using plasmid pYT6 as a template. The primers for this PCR (1 and 2 listed in Table 1 and shown in Fig. 1 A) included restriction sites for *Bam*HI and *Hind*III, respectively. The 5'-flanking fragment was inserted into *Bam*HI- and *Hind*III-digested pOS5 to create pOS7. For the third step, a 1078-bp PCR fragment composed of sequences from the 3' terminus of Pp*VPS15*, beginning at nucleotide 2281 of the ORF, was amplified by PCR using plasmid pYT6 as a template. The primers for this amplification (3 and 4 in Table 1 and Fig. 1 A) included sites for *SalI* and *Kpn*I, respectively. The amplified fragment was inserted into *Xho*I- and *Kpn*I-digested pOS7 to create pOS10. The latter plasmid was digested with *Xba*I and *Xho*I, releasing a 4.7-kb fragment comprised of *ARG4* flanked by Pp*VPS15* 5'- and 3'-sequences and was transformed into *P. pastoris* strain GS200 (*his4 arg4*) by electroporation (Liu et al. 1995). Arg⁺ transformants were selected on YND medium plus histidine and screened for ones in which the Pp*VPS15* gene had been deleted (*vps15D*) by testing for temperature sensitivity at 37 °C, a known phenotype of *S. cerevisiae* *vps15D* strains (Herman et al. 1991 a). Temperature-sensitive strains were collected and further examined to confirm deletion of Pp*VPS15*. For this, genomic DNAs were isolated from transformants and used as a template in PCR reactions with two sets of oligonucleotide primers (Table 1, Fig. 1 A). One set was composed of a primer that was complementary to sequences in the 3' flanking region of the Pp*VPS15* ORF that were present in the wild-type gene of Pp*VPS15* and another which hybridized to sequences in the 5' region of, but absent from, the *VPS15D* allele (primers 5 and 3 in Table 1). The other set of primers contained the same 3'-flanking sequence primer described above and a second primer that was complementary to a sequence in *ARG4* (primers 5 and 4 in Table 1). The Southern-blot of wild-type and *VPS15D* strains was performed using the Southern Light DNA Detection kit from Tropix (Bedford, Mass.) as recommended by the supplier. As a hybridization probe, a 2.1-kb DNA fragment containing the Sc *ARG4* gene was simultaneously amplified by PCR and labelled with biotin using the Multiprimer DNA Labelling System kit from Life Technologies (Gaithersburg, Md.) as recommended. To examine colonies for their ability to degrade the peroxisomal enzyme AO, colonies were replica-plated onto YNM plates and incubated for 1.5 days to induce AO. They were then further replica-plated onto sets of YND or YNE plates to induce peroxisomal degradation. After incubation for 8 and 12 h, respectively, AO activity was visualized in colonies by overlaying plates with an AO-activity reaction mixture plus digitonin to permeabilize the cells (Titorenko et al. 1995).

Biochemical methods. Preparation of crude extracts was performed as described previously (Waterham et al. 1994), except that a protease-inhibitor cocktail was added to all buffers (Complete, Boehringer Mannheim, FRG). Protein concentrations were determined as described by Bradford (1976) using bovine serum albumin as a standard. AO activity was assayed as described by Verduyn et al. (1984). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described (Laemmli 1970). Western blotting was performed using the Protoblot

Table 1 PCR primers used in this study

Name	Sequence
1	5'-CgAAgCTTTTACgCTAgAggAATggCgATg-3'
2	5'-ATggATCCAAATCCCAgATCTCCACACTgg-3'
3	5'-AAACTCCTTATGATTCTgC-3'
4	5'-TAATTAgAgAgAggggAggAAC-3'
5	5'-AAATACTgTTgATCCggTg-3'
6	5'-ACgCgTCgACTCAAAGATTggggAATCgg-3'
7	5'-CggggTACCAgATCATAgAAgTCCAAAATACC-3'
8	5'-CTTTgATTgAgAgATATgC-3'
9	5'-gATAAAAgAgTCATTCTCTgg-3'
10	5'-TgCgTTgATgCAATTTCTAT-3'

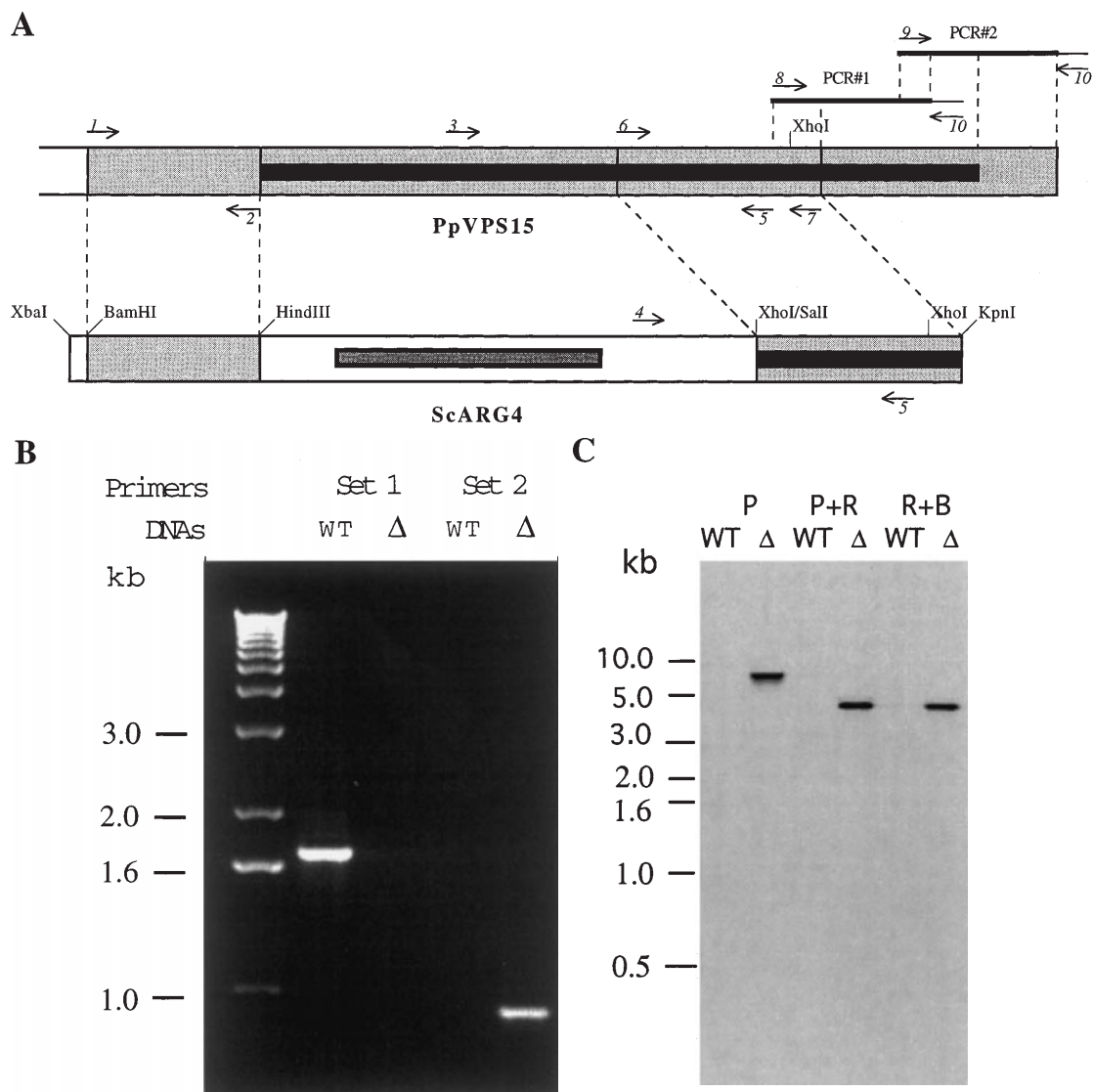


Fig. 1 A–C Isolation of the *P. pastoris VPS15* gene and construction of a *vps15D* strain. **A** Schematic representation of the *VPS15* locus and the *vps15D*-allele PCR primers are shown as arrows with numbers corresponding to the primers listed in Table 1. **B** agarose gel showing fragments generated by PCR with genomic DNAs from *P. pastoris* GS200 (WT) or a *vps15D* strain (Δ) as templates using wild-type *VPS15*-specific primers 3 and 5 or *vps15D*-specific primers 4 and 5 (see A and Table 1). The *VPS15*-specific primers amplified a 1722-bp fragment from WT DNA but no fragment from *vps15D* DNA. Conversely, the *vps15D*-specific primers amplified a 980-bp fragment from *vps15D* DNA but no fragment from WT DNA. The size of selected marker fragments from the 1-kb ladder are shown at the left. **C** Southern blot showing genomic DNAs of the wild-type (WT) and *Ppyps14D* strains (Δ) digested with selected restriction enzymes and hybridized with a labelled probe composed of sequences from the *S. cerevisiae ARG4* gene. The restriction enzyme abbreviations are: *EcoRI*, R; *PvuII*, P; and *BamHI*, B

immunoblotting system (Promega, Madison, USA) and polyclonal antibodies raised against *H. polymorpha* AO, which cross react with *P. pastoris* AO.

Electron microscopy. Cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously

(Waterham et al. 1994). Immunolabelling was performed on ultrathin sections of Unicryl-embedded cells using specific antibodies against *H. polymorpha* AO and goat anti-rabbit antibodies conjugated to 15-nm gold particles (Amersham, UK) according to the instructions of the manufacturer.

Results

Isolation of a *P. pastoris* homologue of *VPS15*

Previously, we cloned the *H. polymorpha PDD1* gene (Kiel et al. 1999) and discovered that it is highly homologous to *S. cerevisiae VPS34*, a gene required for vacuolar protein sorting (Herman and Emr 1990) and endocytosis (Munn and Riezman 1994). In *S. cerevisiae*, ScVps34p function has been shown to be dependent on activation by the protein kinase ScVps15p (Stack et al. 1995 a,b). This led us to speculate that a Vps15p homologue may also be necessary for peroxisome degradation. An opportunity to test this hypothesis was afforded by the presence of the probable homologue of

VPS15 adjacent to a previously cloned gene *PEX2* in the related methylotrophic yeast *P. pastoris* (Waterham et al. 1996). Specifically, 3358 bp of the 5'-terminus of the putative *P. pastoris VPS15* gene (Pp*VPS15*) existed 130 bp downstream from the termination codon of *PEX2* in the *P. pastoris* library plasmid pYT6. To isolate the remaining 3'-terminal portion of the Pp*VPS15* gene, a *P. pastoris* genomic library was subjected to two rounds of chromosome walking by the PCR method using primers complementary to the cloned portions of the gene and to the library vector as detailed in Materials and methods (Fig. 1 A).

The complete open reading frame of the gene was found to be 4023-bp long and to encode a polypeptide of 1340 amino acids. The predicted primary sequence of PpVps15p shared 32% identity and 50% similarity with that of ScVps15p (Herman et al. 1991 a) and 29% identity and 49% similarity with that of the putative human *VPS15* homologue p150 (Panaretou et al. 1997).

Alignment of the predicted primary sequences of the three proteins revealed three strongly conserved regions (Fig. 2). A large N-terminal region of each protein (amino acids 27–305 in PpVps15p) contains the catalytic domain shared by the serine/threonine family of protein kinases (Fig. 2 A; Hanks et al. 1988). A lysine residue in this region (PpK149) is conserved among all known serine/threonine protein kinases while an aspartic-acid residue (PpD147) and a lysine (PpK54) are part of the putative ATP-binding site(s) and are also conserved in both serine/threonine and tyrosine protein-kinases (Hanks et al. 1998). Alteration of any of these three residues in ScVps15p results in a severe reduction in enzymatic activity and mislocalization of vacuolar enzymes (Herman et al. 1991 b). A conserved glycine residue at position 2 in PpVps15p may serve as a myristylation site as it does in ScVps15p. A second conserved region (amino acids 540–670 in PpVps15p) contains a potential transmembrane domain (PpVps15p residues 587–605, Fig. 2 B) and is proposed to play an important role in the function and/or proper intracellular localization of the Vps15p homologues. Finally, the C-terminal 30 amino acids exhibit a high degree of conservation (Fig. 2 C). This region contains a potential phosphorylation site and is essential for ScVps15p function at elevated temperature (Herman et al. 1991 b). Together, the high degree of primary sequence conservation, and particularly that of specific functional domains, support the notion that the cloned gene is most likely a *P. pastoris* homologue of *S. cerevisiae VPS15*.

A Pp*VPS15* deletion strain is defective in glucose- and ethanol-induced peroxisome degradation

To investigate the role of Pp*VPS15* in peroxisome degradation, a *P. pastoris* strain was constructed in which most of the gene was deleted (Pp*vps15D*) (Fig. 1 A). To delete the gene, a plasmid, pSO10, was first constructed in which nucleotides 1 through 2280 (amino-acid

residues 1–760) of the Pp*VPS15* open reading frame had been replaced with a fragment containing the *S. cerevisiae ARG4* gene. A fragment from pSO10 was introduced into *P. pastoris* strain GS200 (*his4 arg4*) and Arg⁺ transformants were selected. Transformed colonies were then screened for ones that were temperature-sensitive for growth at 37 °C, a phenotype of *S. cerevisiae vps15* mutants (Herman et al. 1991 a). Temperature-sensitive strains were further examined by the PCR method to confirm the presence of a properly targeted Pp*vps15D* fragment and the absence of wild-type (WT) Pp*VPS15* sequences (Fig. 1 A,B).

To eliminate the possibility that, in addition to a deleted Pp*VPS15* gene, the Pp*vps15D* strain also harbored a second deleted or disrupted gene, which in fact was responsible for the phenotype of the strain, we examined restricted genomic DNAs from the Pp*vps15D* strain for the number of copies of the Sc*ARG4* gene in the strain. Restriction enzymes were selected that did not cleave the Sc*ARG4* gene so that the number of Sc*ARG4*-derived bands observed in Southern blots would equal the number of Sc*ARG4* gene copies in the strain. As shown in Fig. 1 C, all digests showed only one Sc*ARG4*-derived band demonstrating that only one copy of the Pp*vps15D::ScARG4* construct was present in the strain.

To examine peroxisome degradation and turnover in a Pp*vps15D* strain, cultures of this strain and GS200, a WT control strain, were first grown on methanol medium. The Pp*vps15D* cells grew at the WT rate on methanol and contained peroxisomes that were morphologically indistinguishable from WT, indicating that PpVps15p is not required for the biogenesis of these organelles. In order to analyze peroxisome turnover, Pp*vps15D* and WT cells were again grown in methanol as the sole carbon source to the late exponential growth phase ($D_{663} = 1.5$ – 2.0). Subsequently, 1.0% glucose or 1.0% ethanol was added to the cultures and at selected times culture samples were removed and analyzed. To study the fate of peroxisomes in the cells, crude extracts were prepared and examined for levels of the peroxisomal matrix enzyme AO. The results, presented in Table 2, showed that after addition of glucose to Pp*vps15D* cultures, AO specific activity remained approximately constant (Table 2), whereas in WT control cultures, AO activity decreased to 12% of the initial value within 6 h of glucose addition. Western blots of Pp*vps15D* extracts revealed that the amount of AO protein in the cells also did not decrease significantly with time after addition of glucose, suggesting that peroxisomes were not degraded (Fig. 3). This was confirmed by electron microscopy where the uptake of peroxisomes into the vacuole, the initial event in peroxisome degradation in WT cells, was not observed (data not shown, compare Fig. 4). In the WT control strain, normal AO inactivation and degradation patterns were observed (Table 2; Fig. 3). From these results, we concluded that Pp*vps15D* was defective in glucose-induced peroxisome degradation. Peroxisome degradation was also not observed in methanol-grown cells of the Pp*vps15D* strain after the addition of ethanol

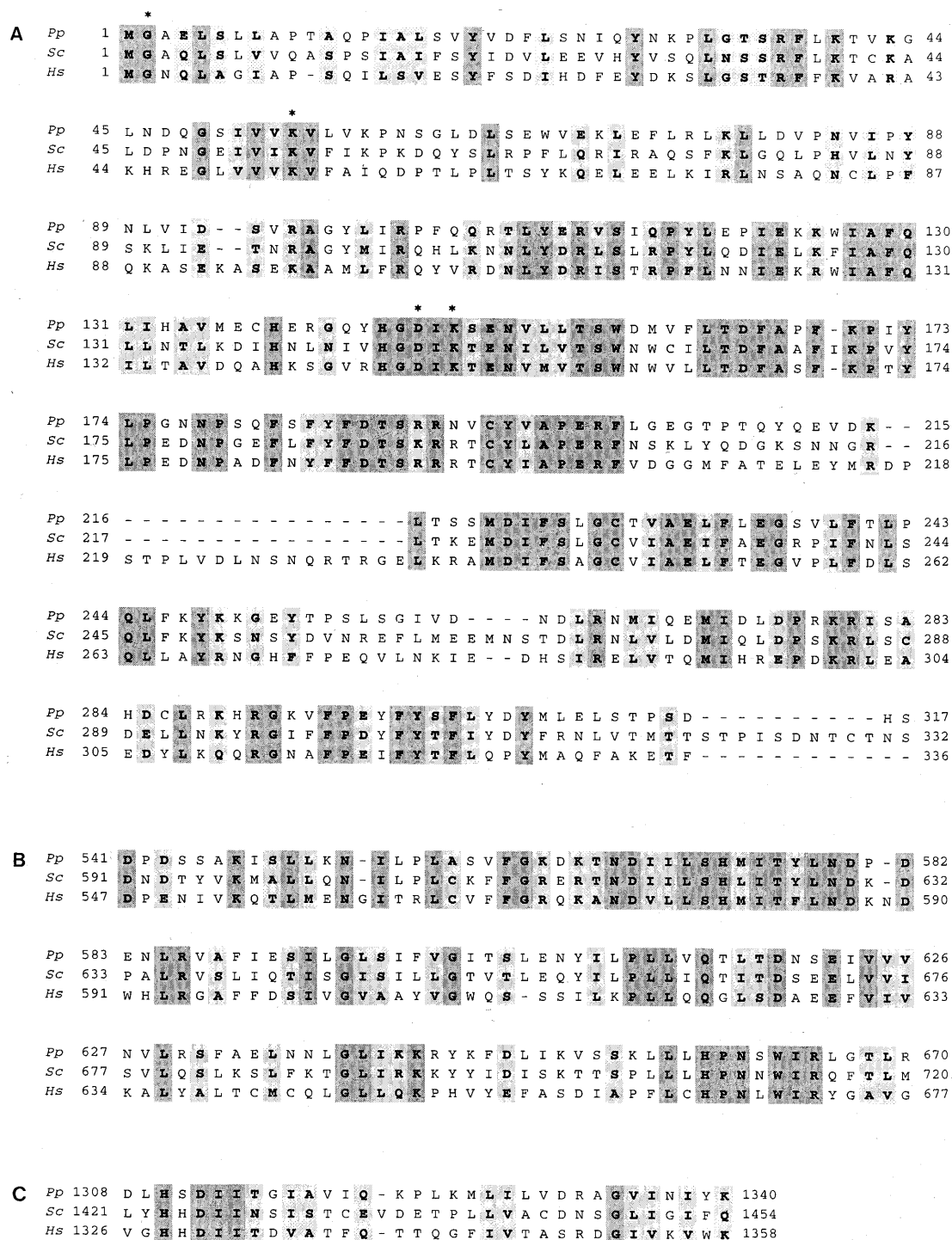


Fig. 2 A–C Alignment of selected regions of *P. pastoris* Vps15p (*Pp*), *S. cerevisiae* Vps15p (*Sc*), and *Homo sapiens* p150 (*Hs*). **A** amino-terminal region including the serine/threonine kinase domain; **B** region with a potential transmembrane domain; and **C** carboxy terminal region. Conserved residues are shown in *bold* with *dark-gray shaded areas* indicating identical residues and *light gray* indicating similar residues. Conserved amino-acid residues in the amino-terminal region (*A*) are PpG2, PpK54, PpD147 and Pp149, and are *highlighted* (*)

(Table 2, Figs. 3 and 4 A). AO activity in *Ppyps15D* cultures decreased slightly, but significantly, upon the addition of ethanol (Table 2). However, we observed no reduction in AO protein levels (Fig. 3) or degradation of peroxisomes (Fig. 4). The decrease in AO activity was most probably due to inactivation of the enzyme. A similar phenomenon is seen in *H. polymorpha pex* mutants. In such cells AO is mislocated to the cytosol and not susceptible to glucose- or ethanol-induced degradation. However, upon addition of excess ethanol the

Table 2 AO activity in *P. pastoris* WT and *vps15Δ* after addition of glucose or ethanol to methanol-grown cells

Strain	Time (h)	Substrate ^a	Optical density (D ₆₆₃)	AO activity U · mg ⁻¹	Activity (%) corrected for growth
WT	0	Glucose	2.0	3.3	100
	3	Glucose	3.0	0.7	32
	6	Glucose	3.9	0.2	12
<i>vps15Δ</i>	0	Glucose	1.6	1.3	100
	3	Glucose	2.1	1.0	101
	6	Glucose	2.4	0.9	101
WT	0	Ethanol	2.0	3.3	100
	3	Ethanol	2.3	2.4	84
	6	Ethanol	2.6	1.4	55
<i>vps15Δ</i>	0	Ethanol	1.6	1.3	100
	3	Ethanol	1.8	1.1	95
	6	Ethanol	1.9	0.8	71

^a Glucose (1%) or ethanol (1%) were added to methanol-grown cells of *P. pastoris* WT or *vps15Δ*. Samples were taken at the indicated time points

AO enzyme is partially inactivated (Van der Klei et al. 1991 b), possibly by the product of ethanol oxidation, acetaldehyde.

In the *P. pastoris* WT cells, peroxisome degradation occurred upon addition of ethanol albeit at a slower rate relative to the cells exposed to glucose (Table 2; Figs. 3, 4 B).

Discussion

In this paper, we describe the cloning and characterization of PpVPS15, a *P. pastoris* gene homologous to *S. cerevisiae* VPS15. The rationale behind this work began with our observation that the *H. polymorpha* PDD1 gene which is required for selective peroxisome degradation in this yeast is homologous to *S. cerevisiae* VPS34 (Kiel et al. 1999). The *S. cerevisiae* VPS34 protein product, ScVps34p, is required for proper sorting of

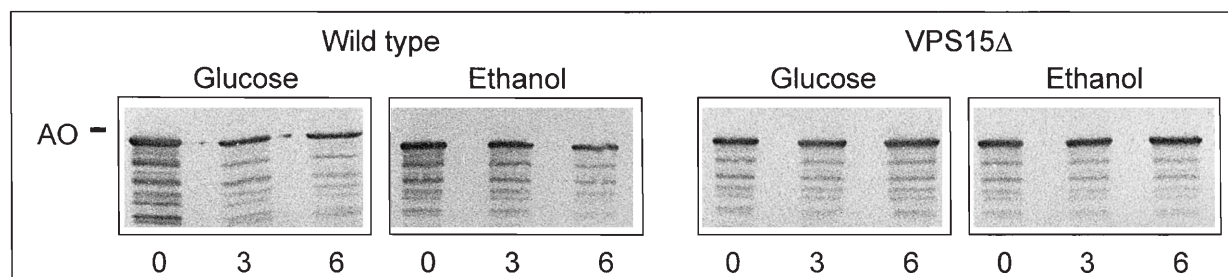
vacuolar proteases and for endocytosis (Hermann and Emr 1990; Stack et al. 1993; Munn and Riezmann 1994) and is dependent on ScVps15p for activation (Stack et al. 1995 a,b). From this, we postulated that Vps15p homologues might also be essential for selective peroxisome degradation. Our data strongly support this notion. Hence, a protein complex, similar to *S. cerevisiae* Vps15p/Vps34p, may be involved in (the regulation of) selective peroxisome degradation in methylotrophic yeasts.

In *P. pastoris*, the initial steps in the mechanism of peroxisome degradation has been shown to be dependent on the compound used as inducer (Tuttle et al. 1993). In methanol-grown cells, the organelles are degraded by a macroautophagic process when the cells are exposed to ethanol, whereas they are degraded by microautophagy upon exposure to glucose. The occurrence of two distinct pathways is supported by the existence of *P. pastoris* mutants that are defective in peroxisome microautophagy but continue to degrade the organelles macroautophagically. In *H. polymorpha*, peroxisomes are invariably degraded by macroautophagy independent of the inducer (Veenhuis et al. 1983; Titorenko et al. 1995). Moreover, all *H. polymorpha* pdd mutants are affected in both glucose- and ethanol-induced peroxisome degradation.

Deletion of PpVPS15 leads to an early block in peroxisome degradation at a point prior to uptake of the organelles by the vacuole. These observations suggest that PpVPS15 is required for an initial step in peroxisome degradation in both micro- and macro-autophagic processes. This is the same phenotype we observed with a *H. polymorpha* pdd1D (*Hpvps34D*) strain (Kiel et al. 1999), supporting the notion that, as in vacuolar sorting, the products of these genes function together in peroxisome autophagy.

The *S. cerevisiae* VPS34 gene (ScVPS34) was first identified among mutants defective in vacuolar protein sorting (Herman and Emr 1990). Later, Scvps34 mutants were independently found among mutants defective in endocytosis (end12 mutants; Munn and Riezman 1994), indicating that sorting of proteases to the vacuole and late steps in endocytosis require at least some common gene products (Munn and Riezman 1994). Disruption of either ScVPS34 or ScVPS15 results in a set of specific phenotypes including temperature-sensitive growth, defects in osmoregulation, disturbed vacuolar segregation, and missorting of multiple soluble vacuolar hydrolases.

Fig. 3 Western-blot analysis of AO degradation in WT and *vps15Δ*. Glucose or ethanol was added to exponential methanol cultures of WT or *vps15Δ*. Samples were taken at t = 0, 3 or 6 h after the addition of glucose or ethanol. Western-blot was prepared from crude cell extracts and decorated with anti-AO antibodies. The lower-molecular-weight bands represent AO degradation products. Equal amounts of protein were loaded per lane



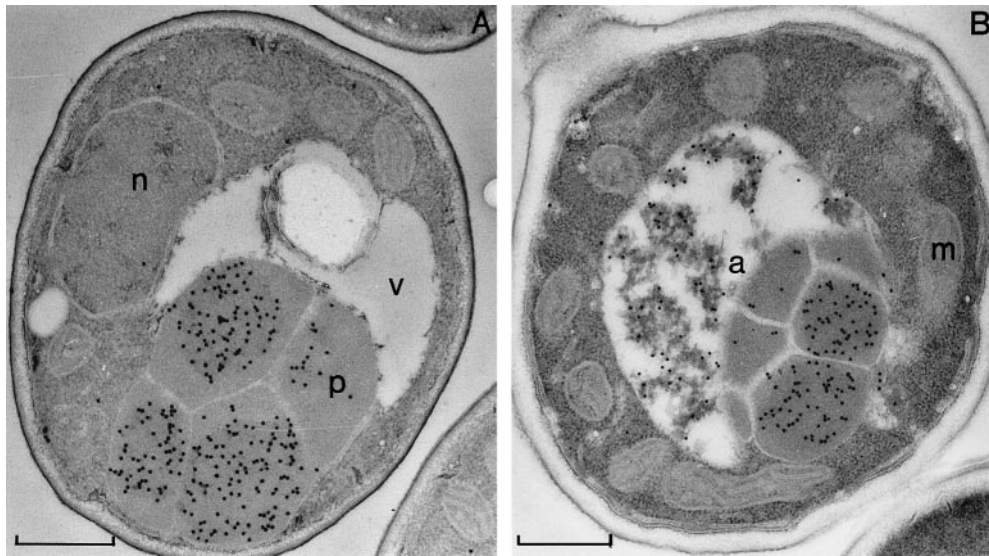


Fig. 4 Immunocytochemical localization of AO in *P. pastoris vps15D* (A) or WT cells (B). Ethanol was added to cultures growing to the mid-exponential growth phase on methanol. Samples were taken after 2 h of incubation. In the *vps15D* mutant AO labelling is confined to the peroxisomes (A). However, in WT cells AO protein is observed both in peroxisomes and in the vacuole/autophagosome (B). a – autophagosome; m – mitochondrion; n – nucleus; p – peroxisome. The bar represents 0.5 mm

ScVPS15 encodes a protein (ScVps15p) that belongs to the serine/threonine family of protein kinases, whereas ScVps34p is a phosphatidylinositol 3-kinase. The proteins form a complex that is associated with the cytosolic face of an as yet unidentified, intracellular membrane. The function of ScVPS15p is to recruit ScVps34p to the membrane and is required for the activation of ScVps34p. Formation of activated ScVps34p at the membrane leads to phosphorylation of the phosphatidylinositol (PtdIns) molecules in the lipid bilayer. Localized patches of PtdIns-3-P in membranes are then thought to recruit, or activate, other effector molecules (De Camilli et al. 1996). For vacuolar protein sorting, these patches are believed to facilitate the binding of coat proteins that are essential for the generation of transport vesicles containing specific vacuolar hydrolases (De Camilli et al. 1996).

The ScVPS34- and ScVPS15-like genes from *H. polymorpha* and *P. pastoris*, respectively, most likely represent the functional homologues of the *S. cerevisiae* genes. Like the corresponding bakers yeast mutants, the *H. polymorpha vps34D* and *P. pastoris vps15D* strains are temperature-sensitive for growth. Furthermore, the *H. polymorpha vps34D* strain is defective in sorting of the vacuolar protease carboxypeptidase Y (Kiel et al. 1999). However, further studies aimed at comparing the properties of the *S. cerevisiae* and methylotrophic yeast mutants are needed to confirm that the affected genes are indeed functional homologues.

A simple explanation for the peroxisome degradation defects observed in the Ppvps15D and Hpvps34D strains is that these mutants have reduced vacuolar proteolytic

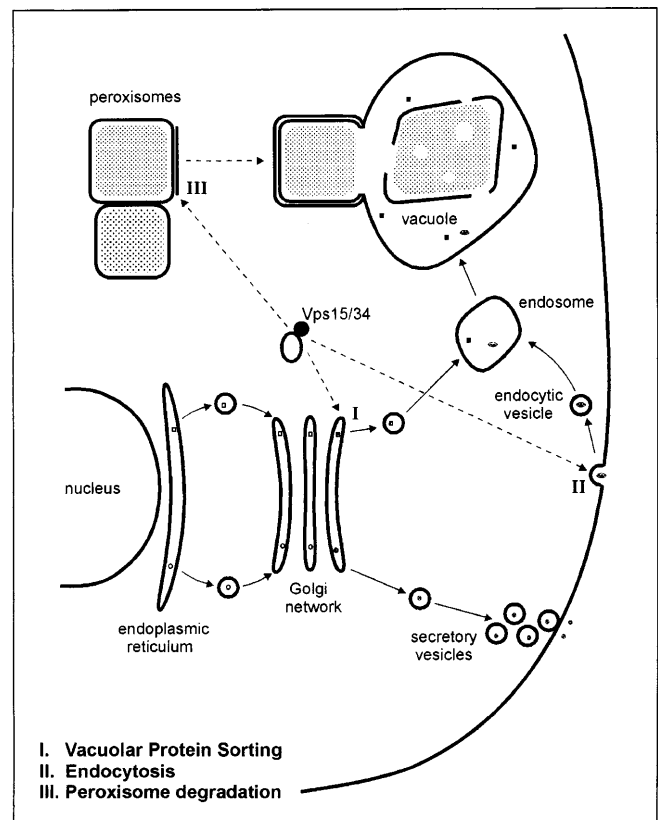


Fig. 5 Multiple roles for the Vps34p/Vps15p complex. Schematic representation of the sites of action of the putative Vps34p(Pdd1p)/Vps15p phosphatidylinositol 3-kinase (PI3K) complex in the yeasts *H. polymorpha*, *P. pastoris* and *S. cerevisiae*. Components of this complex have been demonstrated to play an essential role in the sorting of soluble vacuolar proteins from a late Golgi compartment to early endosomes (I), to be involved in the endocytic pathway (II), and to be essential in selective pexophagy during carbon-catabolite inactivation (III). The complex is thought to reside on vesicular structures. It is believed that the PI3 K activity of the complex tags transport proteins to the endosome (I, II) or to initiate the sequestration process during pexophagy (III)

activities as a byproduct of their vacuolar protein-sorting defects. However, this explanation is not likely since peroxisomes should then be observed to accumulate in vacuoles, as seen in mutants defective in vacuolar proteases (Tuttle and Dunn 1995; Chiang et al. 1996; Sakai et al. 1998). A more satisfactory explanation, and one that is consistent with their proposed roles in vacuolar protein sorting and endocytosis, is that Vps15p and Vps34p are involved in tagging the organelles by the phosphorylation of PtdIns molecules for delivery to the vacuole. Thus, it appears that the Vps34p/Vps15p complex plays an important role in each of the numerous vacuolar delivery pathways. A schematic representation of these is given in Fig. 5.

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